

**Methods and Compositions for Modulating Proteins Modified
in Preconditioning Against Ischemia/Hypoxia**

Introduction

5 This patent application claims the benefit of priority from U.S. Provisional Application Serial No. 60/463,139, filed April 14, 2003, which is herein incorporated by reference in its entirety.

10 **Field of the Invention**

 Modifications to various proteins including, but not limited to, proteins involved in the tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OxPhos) pathways, calcium (Ca^{2+}) handling, and/or chaperoning, as well as
15 proteins selected from aldehyde dehydrogenase, NG-dimethylarginine dimethylaminohydrolase (DDAH) and the RNA binding protein regulatory subunit DJ-1, have been identified which occur during pharmacological
preconditioning, a treatment which mimics many aspects of
20 classical ischemic preconditioning or hypoxia including protection of a cell, tissue or organ from damage resulting from prolonged periods of ischemia, hypoxia, ischemia/reperfusion, hypoxia/reperfusion or any other event or agent that causes or promotes cell death (necrosis
25 or apoptosis) or injury. The present invention provides methods or events and compositions for modulating one or more of these preconditioning proteins as well as methods for identifying new compositions and methods or events capable of modifying one or more of these preconditioning
30 proteins. The present invention also provides methods or events and compositions for priming a cell for preconditioning, for preconditioning a cell, tissue or organ, and/or for modulating preconditioning of a cell tissue or organ, as well as methods for identifying new
35 compositions and methods or events for priming a cell for preconditioning, for preconditioning a cell, tissue or organ, and/or for modulating preconditioning of a cell, tissue or organ based upon the ability of the composition

to modify one or more of these preconditioning proteins.
In addition, the present invention provides methods for
diagnosing effective preconditioning and monitoring
preconditioning of a cell, tissue or organ based upon
5 detection of one or more of these preconditioning proteins.

Background of the Invention

Oxidative metabolism is the means by which all
eukaryotic cells convert extracellular substrate (in the
10 form of carbohydrates, lipids, and some amino acids) into
adenosine 5'-triphosphate (ATP) to meet cellular energy
demands. Under normal conditions, ATP production meets
demand, thus pathways involved in ATP synthesis are well
controlled and respond quickly to changes in energy
15 requirements. This process of metabolizing substrate into
ATP can be divided into a three stage process (as described
by Jafri et al. Annu. Rev. Biomed. Eng. 2001 3:57-81). In
the first stage, energy substrate is delivered across the
mitochondrial inner membrane to the tricarboxylic acid (TCA
20 or Krebs) cycle via glycolysis of carbohydrates, β -
oxidation of fatty acids, and conversion of amino acids
into pyruvate or TCA cycle intermediates. In the second
stage, the TCA cycle in the mitochondrial matrix links
glycolysis to oxidative phosphorylation (OxPhos) through
25 decarboxylation of pyruvate to acetyl-coenzyme A (CoA) and
the complete oxidation of acetyl-CoA to CO₂ (see Figure 1).
In the third stage, oxidative phosphorylation (OxPhos)
oxidizes reducing equivalents produced by the TCA cycle via
the electron transport chain as a means of establishing a
30 large electrochemical proton gradient across the
mitochondrial inner membrane (see Figure 2 for schematic of
the OxPhos system and the interrelationship to the TCA
cycle). This proton motive force is subsequently used by
ATP synthase to couple the flow of protons into the
35 mitochondrial matrix with the phosphorylation of adenosine
5'-diphosphate (ADP) to form ATP.

During ischemia or hypoxia, normal oxidative
metabolism is jeopardized, with the risk of cell injury and

cell death increasing with increased duration of ischemia or hypoxia. Sudden occlusion of an artery results in oxygen deprivation to the region downstream of the occlusion. This is followed by physiological and metabolic changes that
5 begin within seconds, with the following sequence of events known to occur in a well-studied model of coronary occlusion in dogs (Kloner et al. Circulation 2001 104:2981-2989).

As taught by Kloner et al. (Circulation 2001 104:2981-
10 2989), after about 8 seconds of decreased arterial blood flow, the O₂ trapped in the tissue as oxyhemoglobin and oxymyoglobin has been consumed and energy metabolism shifts from aerobic or mitochondrial metabolism to anaerobic glycolysis. Effective contractions begin to decrease and
15 finally stop, and the myocardium stretches, instead of shortening, with each systole. The membrane potential decreases and electrocardiogram (ECG) changes can be observed.

Kloner et al. (Circulation 2001 104:2981-2989) also
20 teaches that the energy demands of myocytes greatly exceed the supply from anaerobic glycolysis and reserves of high-energy phosphate (HEP). Thus, tissue ATP and creatine phosphate decrease and ADP and inorganic phosphate and hydrogen ions begin to accumulate. Creatine phosphate, a
25 major reserve source of HEP, decreases rapidly with 90% being exhausted after 30 seconds of ischemia. ATP levels decrease more slowly with approximately 20% to 25% of the ATP present at the onset of ischemia still being present late in the reversible phase of ischemia. Approximately
30 80% of the new HEP in zones of severe ischemia is produced by anaerobic glycolysis. Glucose-1-P from glycogenolysis serves as the substrate in anaerobic glycolysis since little glucose is present in the extracellular fluid. The process of anaerobic glycolysis generates 3 μ mol HEP per
35 μ mol of glucose-1-P (Kloner et al. Circulation 2001 104:2981-2989).

After about 10 minutes of ischemia, intracellular pH decreases to 5.8-6.0, and the load of intracellular

osmotically active particles, lactate, inorganic phosphate, creatine, etc, increases markedly (Kloner et al. Circulation 2001 104:2981-2989). Only a modest increase in intracellular H₂O is observed, however, since relatively
5 little H₂O is available in the extracellular space of severely ischemic tissue. This edema can be viewed by transmission electron microscopy as an increase in the sarcoplasmic space.

The adenine nucleotide pool is also degraded as the
10 ADP formed from the action of ATPases accumulates and the rephosphorylation of ADP to ATP via anaerobic glycolysis is slowed by acidosis and lactate and the diffusion of adenosine into the extracellular fluid. Various substances including bradykinin, opioids, norepinephrine, and
15 angiotensin, are also released into the extracellular fluid during the first few minutes of ischemia. Like adenosine, these agents bind to receptors on myocytes and stimulate intracellular signaling system responses. These reactions occur quickly. For example, phosphorylase is activated only
20 a few seconds after the onset of ischemia by the norepinephrine that is released from intramyocardial sympathetic nerve endings as a response to ischemia (Kloner et al. Circulation 2001 104:2981-2989).

Calcium is involved in, and is essential for,
25 triggering contraction. Its balance is critical to the cell, however, as overload of Ca²⁺ causes hypercontraction, precipitation of Ca²⁺ phosphate in the mitochondria, and ultimately cell death. In the isolated perfused heart, late in the reversible phase of ischemia, intracellular
30 ionic Ca²⁺ rises slightly (Kloner et al. Circulation 2001 104:2981-2989). This has been difficult to confirm, however, *in vivo*.

Restoration of arterial flow, also known as
reperfusion, to ischemic living myocardium results in
35 restoration of aerobic metabolism and salvage of the ischemic myocytes (Kloner et al. Circulation 2001 104:2981-2989). Upon reperfusion, the tissue develops reactive hyperemia caused by a 400% to 600% increase in blood flow.

This increased blood flow reaches a peak during the first 5 minutes of reperfusion and then declines to normal control levels over the next 10 to 15 minutes. Excess O₂-derived free radicals also appear during the first minute of
5 reperfusion and peak approximately 4 to 7 minutes after the onset of reperfusion. Generalized mitochondrial and cell swelling can be observed via electron microscopy during this period. ECG changes observed during ischemia disappear after 1 to 2 minutes of arterial reperfusion and
10 a large amount of ATP is produced via rephosphorylation of the ADP and AMP that accumulated while the tissue was ischemic. Lactate decreases to control levels and the pH of the tissue returns to normal levels approximately 0.5 to 2 minutes after reperfusion (Kloner et al. Circulation 2001
15 104:2981-2989).

Preconditioning (PC), a phenomenon which exists in all species examined, including humans, is a form of protection wherein a brief ischemic or hypoxic episode prevents or reduces the extent of cellular or organ damage, death
20 and/or cellular dysfunction from subsequent prolonged ischemia. PC may also be recruited pharmacologically using agonists such as adenosine and diazoxide. PC may also occur from other events and/or agents causing cell death, damage and/or cellular dysfunction. Preconditioning occurs
25 in various organs and tissues including, but not limited to, myocardium, skeletal muscle, smooth muscle, liver, brain and kidney.

For example, adenosine is released from cells immediately with ischemia and affects organs such as the
30 heart as well as the vascular system through a second messenger signaling cascade triggered by binding to adenosine A₁, A_{2a}, A_{2b} and/or A₃ receptors. In the heart, adenosine affects the intrinsic conducting system (bradycardia and AV block potential arrhythmia). In
35 myocytes it affects the Ca²⁺ current (negative inotropic) and has been proposed to influence the function of mitochondrial K_{ATP} channels-. It can affect the vascular system as well causing vasodilation. Adenosine causes

preconditioning, potentially through activation of protein kinase C (PKC) and modulation of the mitochondrial and/or sarcolemmal K_{ATP} channel (Cohen et al. Annu Rev Physiol 2000 62:79-109), although the underlying mechanism remains
5 controversial.

Summary of the Invention

It has now been found that modifications to a number of proteins, including but not limited to, proteins
10 involved in the TCA cycle, OxPhos pathways, Ca^{2+} handling, and/or chaperoning as well as proteins selected from aldehyde dehydrogenase, NG-dimethylarginine dimethylaminohydrolase (DDAH) and the RNA binding protein regulatory subunit DJ-1, occur during pharmacological
15 preconditioning, a treatment which mimics many aspects of classical ischemic preconditioning or hypoxia, including protection of a cell, tissue or organ from damage resulting from prolonged periods of ischemia, hypoxia, ischemia/reperfusion, hypoxia/reperfusion or any other
20 event or agent that causes or promotes cell death (necrosis or apoptosis) or injury. These proteins, namely modified proteins of the TCA cycle, modified proteins of the OxPhos pathways, modified Ca^{2+} handling proteins, modified chaperoning proteins and modified aldehyde dehydrogenase,
25 modified NG-dimethylarginine dimethylaminohydrolase (DDAH) and modified RNA binding protein regulatory subunit DJ-1 are referred to herein as "preconditioning proteins".

Accordingly, an aspect of the present invention relates to methods for identifying new compositions,
30 methods or events useful in modulating a preconditioning protein and/or in priming a cell for preconditioning and/or inducing preconditioning and/or modulating preconditioning of a cell, tissue or organ.

Another aspect of the present invention relates to
35 methods for diagnosing and/or monitoring in a subject preconditioning and/or ischemic, hypoxic, ischemia/reperfusion and/or hypoxia/reperfusion conditions and/or the ability of a cell, tissue or organ to survive

injury by monitoring modulation of a preconditioning protein in the subject. One or more of the preconditioning proteins may be detected in a sample of injured tissue as well as in a biological fluid such as, for example, blood, serum, plasma, urine, saliva, bile, mucus, semen or cerebrospinal fluid, obtained from the subject. Diagnosis of an ischemic, hypoxic, ischemia/reperfusion or hypoxia/reperfusion condition can also be performed by comparing levels of a preconditioning protein measured in a subject with levels of the same preconditioning protein in a control. Modifications, as described herein, in a preconditioning protein in the subject as compared to the control, are indicative of an ischemic, hypoxic, ischemia/reperfusion, or hypoxia/reperfusion condition in the subject. In addition, modifications to a preconditioning protein can be monitored in a subject to assess whether a cell, tissue or organ has been subjected to sufficient preconditioning or requires additional preconditioning for protection from cell, tissue or organ injury or death.

Another aspect of the present invention relates to a composition, a method or an event for modulating a preconditioning protein involved in the TCA cycle and/or an OxPhos pathway, and/or Ca^{2+} handling and/or chaperoning and/or a preconditioning protein selected from aldehyde dehydrogenase, NG-dimethylarginine dimethylaminohydrolase (DDAH) and the RNA binding protein regulatory subunit DJ-1, in a cell, tissue or organ, said composition, method or event being one that induces preconditioning.

Yet another aspect of the present invention relates to a composition, method or event for priming a cell, tissue or organ for preconditioning and/or preconditioning a cell, tissue or organ and preventing injury and/or death and/or modulating preconditioning of a cell, tissue or organ by modulating a preconditioning protein.

Brief Description of the Figures

Figure 1 is a schematic representation of the TCA cycle. This diagram shows all the intermediates, with the corresponding enzymes indicated that are responsible for converting between each of the intermediates. All of these processes occur within the mitochondrial matrix, and all enzymes are found within the matrix, except for succinate dehydrogenase, which is embedded in the inner mitochondrial membrane. Succinate dehydrogenase also functions as Complex II of the OxPhos system (described in Figure 2).

Another important enzymatic process associated with the TCA cycle that is not responsible for conversion between intermediates is that of pyruvate dehydrogenase, which converts pyruvate to acetyl CoA, the initial input substrate for TCA cycle functioning. Acetyl CoA entry is shown in the conversion of oxaloacetate to citrate, at the top of the figure.

Figure 2 is a schematic representation of the oxidative phosphorylation system complexes of the inner mitochondrial membrane showing their interrelationship to the TCA cycle. The schematic shows: (1) the two major sources of the TCA cycle substrate, acetyl CoA, derived from either fatty acid or pyruvate; (2) where acetyl CoA feeds into the TCA cycle and the reducing equivalents NADH and FADH_2 feed into OxPhos complexes I and II in order to facilitate the reduction of O_2 to H_2O ; (3) the five protein complexes (I-V) that comprise the OxPhos system; and (4) the production of ATP that occurs from complex V as a result of both electron transport that initiates from either complex I or II, then proceeds through III and IV to fully reduce O_2 to H_2O , with the associated H^+ transport that occurs through complexes I, III, and IV. The outer and inner mitochondrial membranes are indicated, showing the position of the OxPhos complexes within the inner mitochondrial membrane. The OxPhos complexes labeled I-V are: (I) NADH ubiquinone oxidoreductase; (II) succinate dehydrogenase; (III) ubiquinone cytochrome c oxidoreductase; (IV) cytochrome c oxidase; and (V) ATP synthase. Other molecules are labeled as follows: CoQ is

ubiquinone, cyt C is cytochrome c, and the TCA cycle intermediates are abbreviated for simplicity (C = citrate; IC = isocitrate; α -KG = α -ketoglutarate; S = succinate; F = fumarate; M=malate; OAA = oxaloacetate).

5 Figure 3 shows a schematic of the multi-tiered two-dimensional gel electrophoresis approach used for protein separation and analysis. The whole tissue gel (top) shows the complexity of attempting to homogenize and resolve all proteins simultaneously. To simplify the ventricular
10 myocyte protein profile and to facilitate subsequent detection and analysis of protein changes induced by adenosine and diazoxide preconditioning, a pH-dependent sequential subfractionation was carried out to first obtain two separate subproteomes, the HEPES extract at pH 7.4,
15 followed by the TFA extract at pH 2.0 (middle panels). Analysis was further enhanced by then focusing on specific proteins of interest by the application of different pH gradient zoom gels (bottom), to focus on proteins in a specific pH range, and by using multiple protein loads, to
20 analyze either high abundance proteins (at low loads) or to look for changes to low abundance proteins (at high protein loads).

Figures 4A through 4C show identical regions of silver-stained two-dimensional gels to indicate the intra-
25 gel positions of protein spots that have been identified as changing between pharmacologically preconditioned and untreated rabbit ventricular myocytes from 2-D gels of equivalent protein loads from extracts of each treatment (A=adenosine; C=control; D=diazoxide). For each protein,
30 its location within the adjacent gel image, if present, is indicated by a box. Modified proteins are grouped by cellular function into the following categories: TCA cycle, chaperones, calcium handling (Figure 4A); oxidative phosphorylation (Figure 4B); late preconditioning, and two
35 examples of unmodified proteins included for comparison (Figure 4C). The graphs to the right of the gel images indicate the relative abundance of the protein for each treatment (again, A=adenosine; C=control; D=diazoxide), and

spots which show statistically significant change relative to the corresponding control, are indicated (* $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$, using a two-tailed student's t test to analyze one gel per rabbit, with $n=4$ rabbits per treatment).

Figures 5A through 5C are gels showing the position of all proteins identified, including ones that do change and ones that do not, in the process of analysis for proteomic modifications by either adenosine or diazoxide preconditioning. Figure 5A shows a Coomassie stained gel of 400 μg of rabbit ventricular myocytes HEPES extract focused between pH 4-7 and resolved by 12.5% SDS-PAGE. Figure 5B shows a silver stained gel of 200 μg of rabbit ventricular myocytes HEPES extract focused between pH 6-9 and resolved by 10% SDS-PAGE. Figure 5C shows a silver stained gel of 250 μg of rabbit ventricular myocytes HEPES extract focused between pH 3-10 and resolved by 12.5% SDS-PAGE. On each gel the protein spots are numbered. Table 1, *infra*, lists all proteins identified from the three 2-D gel separation methods, with the proteins numbered in accordance with the numbering of spots in the gel images.

Figure 6 shows a functional classification tree containing all proteins that have been identified by proteomic analysis. Proteins that are not modified by either drug (relative to controls) have white backgrounds. Proteins that do show significant change relative to controls are indicated as follows: proteins affected by adenosine are indicated by darker bordering; proteins affected by diazoxide are indicated by grey backgrounds; and proteins modified by both adenosine and diazoxide preconditioning are indicated by hatched grey backgrounds.

Detailed Description of the Invention

PC triggers two windows of protection, the first (classical PC) becoming manifest within 15 minutes and lasting 1-3 hours. The rapid onset and short duration of protection afforded by classical PC are likely the result of post-translational protein modifications, as 15 minutes

is unlikely to be a sufficient time period to recruit significant *de novo* transcription and translation, and it has been shown that preconditioning can still be conferred in the presence of protein synthesis inhibitors (Thornton et al. Am. J. Physiol. 1990 259:H1822-5). A second, less effective window, known as late preconditioning, begins after 24 hours and lasts 24 to 72 hours. The effects of this second window have been ascribed to reactive oxygen species, *de novo* protein synthesis resulting from altered gene regulation and/or expression, and post-translational modifications (Bolli, R. Circ. Res. 2000 87:972-83). Regulation of protein processing and/or turnover may also be responsible for modulation and/or alteration of nascent and/or functional protein quantities in this second window.

Using a multi-tiered proteomic approach that relied on a sequential subproteomic fractionation, followed by resolution of proteins across a variety of pH gradients and protein loads (see the approach schematic, Figure 3), a large number of significant protein modifications occurring during either adenosine or diazoxide preconditioning have now been identified (see Figure 4). In particular, it has now been found that proteins of the TCA cycle and OxPhos and Ca^{2+} handling pathways, chaperone proteins and proteins selected from aldehyde dehydrogenase, NG-dimethylarginine dimethylaminohydrolase (DDAH) and the RNA binding protein regulatory subunit DJ-1 are modified during preconditioning. These protein modifications are believed to underlie the beneficial effects on organs of preconditioning. These modified proteins may provide for protection of cells against an ischemic or hypoxic event or may prime the cell to protect itself from an ischemic or hypoxic event.

By "prime" or "priming" as used herein it is meant that the modification to the protein leads to or results in: (1) a change via cross-talking, a feed-back mechanism and/or a signaling mechanism which ultimately effects the first window of preconditioning, the second window of preconditioning or both windows of preconditioning of a

cell; or (2) a change in function of the protein complex or pathway of which the modified protein is a member. For example, the protein may be a subunit of an enzyme that has had its function altered, without necessarily showing a change in cross-talk, feed-back, or signaling as described above.

By "preconditioning protein" as used herein it is meant a protein whose modification is observed during or following preconditioning. Exemplary preconditioning proteins identified herein are preferably modified mitochondrial proteins. More preferably the preconditioning protein is involved in Ca^{2+} handling, the TCA cycle, chaperoning, and/or the OxPhos pathway or is a protein selected from aldehyde dehydrogenase, NG-dimethylarginine dimethylaminohydrolase (DDAH) or the RNA binding protein regulatory subunit DJ-1. Use of the term "protein" herein, is meant to include full length proteins as well as fragments of the proteins, including but not limited to degradation products, precursors, subunits of proteins and post-translationally modified forms of the proteins.

The present invention provides compositions and methods or events for modulating these preconditioning proteins, priming a cell for preconditioning and/or inducing or modulating preconditioning in a cell, tissue or organ. The present invention also provides methods for identifying new compositions or events capable of modulating these preconditioning proteins, priming a cell for preconditioning and/or inducing or modulating preconditioning in a cell, tissue or organ. In addition, the present invention provides methods for diagnosing and monitoring preconditioning and/or ischemic, hypoxic, ischemia/reperfusion or hypoxia/reperfusion conditions and/or the ability of a cell, tissue or organ to survive injury in a subject based upon detection of one or more of these preconditioning proteins.

For purposes of the present invention, by the term "preconditioning" or "PC" as used herein is meant to be

inclusive of ischemic, hypoxic, and/or pharmacological preconditioning, as well as preconditioning recruited by other events and/or agents causing cell death (necrosis or apoptosis), damage and/or dysfunction.

5 By the term "event" as used herein, it is meant an incident or experience of a cell, tissue or organ resulting in the preconditioning protein modifications observed herein. Examples of such events include, but are in no way limited to, hypoxia, ischemia, glucose deprivation, thermal
10 shock (high or low), alcohol consumption, hemorrhaging, dehydration and sepsis.

By the phrase "protein modification" or "protein modifications" as used herein, it is meant to include changes, in particular increases or decreases in relative
15 protein abundance, as well as changes in type or abundance or direction of post-translational modifications or other chemical adducts. By "post-translationally modified" or "post-translational modification" it is meant to be inclusive not only of phosphorylation of amino acid
20 residues, but also of other chemical adducts. Chemical adducts known in the art relating to post-translational modification of proteins include, but are not limited to, phosphorylation, glycosylation, glycation, myristylation, prenylation, phenylation, acetylation, nitrosylation,
25 oxidation, s-glutathiolation, amidation, biotinylation, c-mannosylation, flavinylation, farnesylation, formylation, geranyl-geranylation, hydroxylation, lipoylation, methylation, palmitoylation, sulphation, gamma-carboxyglutamic acids, N-acyl diglyceride (tripalmitate),
30 O-GlcNAc, pyridoxal phosphate, phospho-pantetheine, pyrrolidone carboxylic acid, ribosylation and ADP-ribosylation. Preferred chemical adducts are phosphorylation, oxidation, glycosylation, myristylation, prenylation, acetylation, nitrosylation, sulphation,
35 ribosylation and ADP-ribosylation. Thus, by "post-translationally modified" it is meant to be inclusive of any of the above chemical adducts and/or any combination thereof to mature proteins as well as precursors and

subunits thereof. Chemical adducts of preconditioning proteins include such post-translational modification of intact preconditioning proteins and of degradation products of preconditioning proteins.

5 The phrase "degradation product" is defined as any fragment of a preconditioning protein. Degradation products can be produced by, for example proteolysis.

By the term "modulate", "modulates" or "modulating" as used herein, it is meant a change, i.e. an increase or
10 decrease in the level of a protein identified herein and/or an increase or decrease in the level of a post-translationally modified form of a protein identified herein, and/or a different type of post-translational modification to a protein identified herein, and/or a
15 change in partitioning of a protein or proteins in the cell.

Protein modifications occurring during preconditioning were identified by a multi-tiered two-dimensional gel electrophoresis approach (see Figure 3) and subsequent
20 analysis of the cytoplasmic and TFA extracts of adenosine and diazoxide-treated isolated cardiomyocytes (n=4) at concentrations capable of invoking preconditioning. This analysis revealed modifications to pathways of oxidative metabolism involving primarily proteins of the TCA cycle, the electron transport chain responsible for OxPhos and
25 energy production (in the form of ATP) within the mitochondria of the cell, Ca^{2+} handling proteins and chaperoning proteins as well as proteins selected from aldehyde dehydrogenase, NG-dimethylarginine
30 dimethylaminohydrolase (DDAH) and the RNA binding protein regulatory subunit DJ-1 (Figure 4). The modified proteins identified herein are referred to as preconditioning proteins.

Preconditioning proteins of the TCA cycle identified
35 as modified include isocitrate dehydrogenase NAD⁺ specific subunit alpha (IDH), dihydrolipoamide succinyltransferase, succinyl CoA ligase [ADP forming] beta chain and protein X (also known as E3 binding protein, of the pyruvate

dehydrogenase (PDH) complex) (Figure 4). PDH is an indirect component of the TCA cycle, since it is responsible for formation of acetyl CoA, which is the substrate that feeds into and is essential for TCA cycle functioning. In particular, pharmacological preconditioning with diazoxide resulted in increases in IDH and post-translational modification of protein X, and a decrease in dihydrolipoamide succinyltransferase. Pharmacological preconditioning with adenosine resulted in an increase of IDH and succinyl CoA ligase [ADP forming] beta chain. It is believed that aconitate hydratase (aconitase), another TCA cycle subunit, may also be a preconditioning protein undergoing changes in post-translation modification during preconditioning.

Preconditioning proteins of the oxidative phosphorylation (OxPhos) system that were modified (see Figure 4) include proteins within Complex I (NADH ubiquinone oxidoreductase), Complex III (ubiquinol cytochrome c oxidoreductase), and Complex V (F_1F_0 ATPase or ATP synthase). In particular, pharmacological preconditioning with diazoxide increased the 23 kDa, 24 kDa, and 30 kDa subunits (mitochondrial precursors) of Complex I, decreased core protein I of Complex III, and in Complex V, it increased the δ chain (mitochondrial precursor) of the F_1 portion, and increased the d chain (mitochondrial precursor) of the F_0 portion. Pharmacological preconditioning with adenosine also decreased core protein I of Complex III and caused increases in the δ chain (mitochondrial precursor) of the F_1 portion, and in the 24 kDa and 30 kDa subunits, (mitochondrial precursors) of Complex I. Also, adenosine induced an increase in the extent of post-translational modification of the β chain of F_1 portion (mitochondrial precursor) of Complex V. The increase in post-translational modification was detected by the presence of two additional spots at the molecular weight of the intact protein, but which are more acidic, in adenosine-treated myocytes subjected to isoelectric focusing in the first

dimension and SDS-PAGE in the second dimension, followed by silver stain or western blot analysis. In contrast, identical extracts from control samples had only a single protein spot.

5 In addition to the TCA cycle and OxPhos protein changes, two chaperone proteins involved in either mitochondrial protein transport or protein complex assembly were also modified by preconditioning (see Figure 4).

One chaperone protein that was modified by both
10 adenosine and diazoxide was metaxin 2, which was found to be reduced in cell extracts treated with either drug. Metaxin 2 is one subunit of a protein complex responsible for the transport of proteins across the outer mitochondrial membrane (Armstrong et al. J Cell Biochem
15 1999 74:11-22). The vast majority of mitochondrial proteins are encoded by the nuclear genome, and following synthesis outside the mitochondria, they must traverse the mitochondrial membranes prior to reaching their final destinations either within the mitochondrial matrix or the
20 inner mitochondrial membrane, as is the case for many of the OxPhos subunits. Metaxin 2 is one of many chaperones that assist in this translocation process.

Another chaperone protein, prohibitin, was modified by adenosine preconditioning. Rather than acting as a
25 transport chaperone, this protein is known to function at the surface of the inner mitochondrial membrane in the intermembrane space (Back et al. Protein Sci. 2002 11:2471-2478), as part of a protein complex that functions in the assembly of mitochondrial respiratory chain (OxPhos)
30 complexes. It does so by binding to and stabilizing newly synthesized mitochondrial subunits against degradation by mitochondrial membrane bound metalloproteases during transport and assembly in the inner mitochondrial membrane (as reviewed by Nijtmans et al. Cell Mol Life Sci 2002
35 59:143-155). Furthermore, it has been suggested that prohibitin function is specifically required in situations of metabolic stress (Nijtmans et al. EMBO J. 2000 19:2444-2451).

Also modified during preconditioning are proteins involved in Ca^{2+} handling or mobilization from the sarcoplasmic reticulum (SR). The SR is a modified endoplasmic reticulum consisting of interconnecting sacs and tubes surrounding the myofibrils (contractile proteins) that contain/store large amounts of Ca^{2+} , which can be released into the cytoplasm to trigger muscle contraction in a process known as Ca^{2+} -induced Ca^{2+} release (CICR). CICR occurs when a small amount of extracellular Ca^{2+} enters the myocyte, which then triggers a large amount of Ca^{2+} release from the SR through the Ca^{2+} release channel, also called ryanodine receptor. Besides the importance for muscle contraction, Ca^{2+} mobilization is important for Ca^{2+} -dependent cellular signaling, and for Ca^{2+} -dependent activation of three TCA cycle dehydrogenases (IDH, PDH, and α -ketoglutarate dehydrogenase).

One Ca^{2+} handling protein modified by preconditioning was the 58 kDa isoform of sarcalumenin, which is present in the SR (see Figure 4). Phosphorylation of cardiac sarcalumenin prevents its binding to the ryanodine receptor (as determined by Hadad et al. J Memb Biol. 1999 170(1):39-49). Thus, it is believed that sarcalumenin plays a role in CICR by regulating ryanodine receptor activity. Sarcalumenin is also present in skeletal muscle where its quantity is affected by chronic low frequency stimulation and hibernation. A decrease in sarcalumenin levels is observed in the HEPES extract of cells preconditioned with diazoxide. This reduction may result from a variety of factors, for instance, a change in phosphorylation state that gives sarcalumenin different affinity for the ryanodine receptor in the presence of diazoxide, or it may simply indicate a difference in absolute quantity arising via differences in extent of protein degradation.

Another protein with an influence on Ca^{2+} mobilization, ADP ribosyl hydrolase, was modified by both adenosine and diazoxide preconditioning (see Figure 4). This protein, which was increased in drug treated extracts relative to matching control extracts, mediates the removal of ADP

ribose moieties from proteins post-translationally modified by ADP ribosylation (as reviewed by Higashida et al. Pharmacology & Therapeutics 2001 90:283-296). The importance of ADP ribosyl hydrolase during preconditioning
5 may relate to one or both of two factors, Ca^{2+} handling and OxPhos Complex I activity, each of which may arise due to their association with metabolism of intracellular nicotinamide adenine dinucleotide (NAD^+).

As reviewed by Higashida et al. (Pharmacology &
10 Therapeutics 2001 90:283-296), NAD^+ metabolism involves a large number of enzymes, one of which is ADP ribosyl hydrolase. One of the major products of NAD^+ metabolism is cyclic ADP ribose (cADPR), which can regulate Ca^{2+} release from the ryanodine receptor, and in particular Type II
15 ryanodine receptors, either by direct interaction with the receptor, or by interacting with FK506, another protein that regulates ryanodine receptor activity (as taught to us by Higashida et al. Pharmacology & Therapeutics 2001 90:283-296). Formation of ADP ribose by ADP ribosyl
20 hydrolase promotes the formation of cADPR from NAD^+ by inhibiting another enzyme, cADPR hydrolase, thereby influencing Ca^{2+} release from the SR (Genazzani et al. Biochem Biophys Res Commun 1996 223:502-507).

Besides the influence on cADPR and SR Ca^{2+} release, ADP
25 ribosyl hydrolase can also influence functioning of the OxPhos system. Complex I of the OxPhos system functions by using NAD^+ as a source of reducing equivalents to initiate a series of oxidation reduction reactions that drive electron transport through the OxPhos system. The
30 significance of ADP ribosyl hydrolase to this process is that the formation of ADP ribose, the by-product of ADP ribosyl hydrolase activity, has been shown to competitively inhibit OxPhos Complex I activity (Zharova and Vinogradov, Biochim Biophys Acta 1997 1320:256-264).

35 Another possibility of the effect of ADP ribosyl hydrolase on Ca^{2+} mobilization is that it may influence release of Ca^{2+} not from the SR, but from mitochondria themselves, via the influence ADP ribosyl hydrolase has on

increasing the abundance of cADPR. As taught by Ziegler (Eur. J. Biochem. 2000 267:1550-1564), cADPR may stimulate the release of Ca^{2+} from mitochondria. Since one of the proposed mechanisms of cell death is by precipitation of Ca^{2+} phosphate in the mitochondria, prevention of
5 mitochondrial Ca^{2+} overload by cADPR may participate in prevention of cell death.

Four additional proteins were also found to be modified by preconditioning (see Figure 4). One of the
10 proteins, previously linked to preconditioning, is HSP27. Dana et al (Circ Res 2000;86:989-997) demonstrated that the preconditioning effect of activating adenosine A1 receptors was accompanied by phosphorylation of HSP27, which is believed to enhance its protective effect. We did not,
15 however, observe a change upon administration of adenosine, but did see an increase following diazoxide treatment.

The other proteins, NG-dimethylarginine dimethylaminohydrolase (DDAH), an RNA binding protein regulatory subunit, also known as DJ-1, and aldehyde
20 dehydrogenase have not previously been linked to preconditioning. All of these proteins were increased in diazoxide-treated cells, while only DJ-1 and aldehyde dehydrogenase increased following treatment with adenosine. DDAH was decreases following treatment with adenosine.

25 DJ-1 has been taught to be converted into a variant having a more acidic pI in response to exogenous oxidative stress or endogenous reactive oxygen species (Bonifati et al. J Mol Med. 2004 82:163-174), suggesting a role for DJ-1 as an antioxidant, or a sensor of oxidative stress. The
30 changes observed during adenosine and diazoxide preconditioning may relate to this pI shift, or may relate to proposed functions of the protein. Although these are still not well understood, Bonifati et al. (J Mol Med. 2004 82:163-174) have proposed that DJ-1 is involved in cellular
35 stress responses at three possible levels: (1) it may directly react to stress signals (e.g., redox changes, misfolded proteins) being an antioxidant and/or a molecular chaperone; (2) it may modulate gene expression of the

stress response at the post-transcriptional level by its known interaction with RNA-binding protein complexes; and (3) it may translocate to the nucleus in response to stress signals, and in so doing, modulate gene expression directly at the transcriptional level.

DDAH is the only protein identified herein which is affected by both diazoxide and adenosine preconditioning that is modified in different directions by the two agents, being increased by diazoxide and decreased by adenosine treatment. This enzyme metabolizes asymmetric dimethylarginine, which is an endogenous inhibitor of nitric oxide synthase (NOS), thereby facilitating the synthesis of nitric oxide. Dayoub et al. (Circulation. 2003 108:3042-3047) demonstrated that DDAH overexpression in transgenic mice increases NOS activity *in vitro* and *in vivo*, leading to physiological effects consistent with increased production of nitric oxide, such as reduced systolic blood pressure, systemic vascular resistance, and cardiac stroke volume. This indicates that metabolism of endogenous asymmetric dimethylarginine plays an important role in regulation of NOS activity, and both nitric oxide and NOS activity are important factors in the late phase of preconditioning, as taught by Dawn and Bolli (Ann NY Acad Sci. 2002 962:18-41).

While many protein modifications by each preconditioning agent were detected and identified, these were by no means all the proteins identified during this study. A total of 112 individual protein spots taken from gels resolved under 3 distinct conditions were identified (Figure 5 and Table 1).

Table 1: Protein species identified by MALDI and MS/MS. Numbers 1-43 correspond to the gel in Figure 5A, numbers 44-63 correspond to the gel in Figure 5B, and numbers 64-112 correspond to the gel in Figure 5C. Proteins existing as multiple species in a gel are indicated by *, while species detected in more than one gel map are indicated by ‡.

Spot #	Name	Identified by:	% coverage, # of peptides sequenced	Accession #	Function
1*‡	ATP synthase beta chain	MALDI	50	P10719	ATP production, oxidative phosphorylation
2*‡			54		
3*‡			60		
4*‡	Alpha-actin, cardiac	MALDI	60	P04270	Myocyte structure, muscle contraction
5*‡			68		
6‡	Tropomyosin, alpha chain	MALDI	45	P09493	muscle contraction
7*‡	Myosin light chain 1	MALDI	62	P08590	
8*‡			58		
9	NADH oxidoreductase 23 kDa subunit	MALDI	43	P42028	ATP production, oxidative phosphorylation
10*‡	Myosin light chain 1	MALDI	2 peptides	P08590	muscle contraction
11‡	Heat shock protein 27	MS/MS	5 peptides	P42929	Stress response
12	ATP synthase subunit d	MS/MS	2 peptides	Q9DCX2	ATP production, Oxidative phosphorylation
13	Thioredoxin-dependent peroxide reductase	MS/MS	2 peptides	Q06830	Free radical metabolism
14‡	NADH ubiquinone oxidoreductase 24 kDa subunit	MALDI	35	Q9D6J6	ATP production, Oxidative phosphorylation
15*‡	Myosin light chain 2 (ventricular/cardiac isoform)	MALDI	60	P51667	muscle contraction
16*‡			48		
17	ATP synthase delta chain	MS/MS	5 peptides	P30049	ATP production, Oxidative phosphorylation
18	Cytochrome c oxidase polypeptide Va	MALDI	85	P00426	
19	Fatty acid binding protein	MALDI	70	P10790	Intracellular transport of fatty acids
20‡	Pyruvate dehydrogenase E1 beta subunit	MALDI	36	P11177	Glycolysis, ATP production
21	NG, NG – dimethylarginine dimethylaminohydrolase	MALDI, MS/MS	30, 2 peptides	O94760	Amino acid modification, NO synthase regulation
22*	Isocitrate dehydrogenase subunit alpha NAD+ specific	MALDI	37	P50213	TCA acid cycle, ATP production
23*			37		
24*	ADP-ribosyl hydrolase	MS/MS	2 peptides	Q8NDY3	ADP-ribose modulation – cell signaling
25*			2 peptides		
26	Succinyl-CoA ligase [ADP forming] beta chain	MALDI	25	O97580	TCA acid cycle, ATP production
27	Succinyl-CoA ligase [GDP-forming] beta chain	MALDI, MS/MS	27 3 peptides	Q96I99	
28*	Dihydrolipoamide succinyl transferase	MS/MS	5 peptides	P11179	
29	Protein disulfide isomerase	MS/MS	5 peptides	P30101	Disulfide bond

	A3				generation/breakdown
30	RNA-binding regulatory subunit (identical to DJ-1)	MS/MS		O14805 (Q99497)	Transcriptional modulation
31*	Dihydrolipoamide succinyl transferase	MS/MS	3 peptides	P11179	TCA acid cycle, ATP production
32*‡	Ubiquinol cytochrome c reductase core protein I	MS/MS	5 peptides	P31930	Oxidative phosphorylation, ATP production
33*‡			5 peptides		
34‡	L-lactate dehydrogenase beta chain	MALDI	35	P07195	Glycolysis, ATP production
35	NADH oxidoreductase 49 kDa subunit	MALDI	40	P17694	Oxidative phosphorylation, ATP production
36*	Dihydrolipoamide succinyl transferase	MS/MS	12 peptides	P11179	TCA acid cycle, ATP production
37	Sarcolumenin, 58 kDa isoform	MALDI	20	P13666	Possible role in ryanodine receptor regulation
38	Aldehyde dehydrogenase	MALDI, MS/MS	19, 10 peptides	P11884	Aldehyde metabolism
39	2-oxoisovalerate dehydrogenase beta subunit	MS/MS	3 peptides	P21953	TCA acid cycle, ATP production
40*‡	Pyruvate dehydrogenase E3 binding protein	MS/MS	5 peptides	O00330	Glycolysis, ATP production
41	Prohibitin	MALDI	69	P35232	Mitochondrial protein complex assembly
42	Metaxin 2	MALDI	31	O88441	Mitochondrial protein transport
43	NADH ubiquinone oxidoreductase 30 kDa subunit	MALDI	44	P23709	Oxidative phosphorylation, ATP production
44*	Aconitate hydratase	MALDI	37	P16276	ATP production, TCA acid cycle
45*			30		
46*	Pyruvate kinase M1 isozyme	MALDI	31	P11974	ATP production, glycolysis
47*			47		
48	Glucose-6-phosphate isomerase	MALDI	34	Q9N1E2	
49‡	ATP synthase alpha chain	MALDI	55	P25705	ATP production, oxidative phosphorylation
50*‡	Fumarate hydratase	MALDI	16	P07954	ATP production, TCA acid cycle
51*‡			22		
52	Creatine kinase M chain	MALDI	60	P00563	ATP regeneration
53	Citrate synthase	MALDI	26	O75390	ATP production, TCA acid cycle
54	Creatine kinase, sarcomeric mitochondrial	MALDI	65	P17540	ATP regeneration
55*	Fructose biphosphate aldolase A	MALDI	67	P00883	ATP production, TCA acid cycle
56*			70		
57*	Isocitrate dehydrogenase [NADP], mitochondrial	MALDI	43	Q04467	
58*			42		
59	Triosephosphate isomerase	MALDI	41	P00939	Glycolysis
60	Guanylate cyclase alpha-2 chain	MALDI	16	Q9WVI4	Cell signaling
61	Malate dehydrogenase	MALDI	41	P08249	ATP production, TCA acid cycle

62	Glyceraldehyde 3-phosphate dehydrogenase	MALDI	48	P46406	ATP production, glycolysis
63	Adenylate kinase Isoenzyme 1	MALDI	55	P00569	ATP/ADP balance
64*†	ATP synthase alpha chain	MALDI	51	P19483	ATP production, oxidative phosphorylation
65*†			51		
66*†			40		
67†	Ubiquinol-cytochrome C reductase complex core protein I	MALDI	34	P31930	
68*†	ATP synthase beta chain	MALDI	50	P10719	ATP production, glycolysis
69†	Pyruvate dehydrogenase E1 subunit	MALDI	34	P11177	
70†	L-lactate dehydrogenase beta chain	MALDI	35	P07195	
71*†	ATP synthase beta chain	MALDI	33	P10719	ATP production, oxidative phosphorylation
72*†			48		
73†	Alpha actin, cardiac	MALDI	54	P04270	Muscle contraction
74†	Tropomyosin, alpha chain	MALDI	43	P09493	
75*†	Myosin light chain 1	MALDI	70	P08590	
76*†		MALDI	62		
77*†	Myosin light chain 2	MALDI	51	P10916	
78*†		MALDI	57		
79*†	Myosin light chain 1	MALDI	59	P08590	
80*†		MALDI	41	P08590	
81*†	NADH ubiquinone oxidoreductase 24 kDa subunit	MALDI	31	P19404	ATP production, oxidative phosphorylation
82*†			52		
83*†	Myosin light chain 1	MALDI	58	P08590	Muscle contraction
84	Cytochrome c oxidase Vb	MS/MS		P10606	ATP production, oxidative phosphorylation
85	Cytochrome c oxidase VIa	MS/MS		Q02221	
86	NADH ubiquinone oxidoreductase 13 kDa subunit	MS/MS		O75380	ATP production, oxidative phosphorylation
87*†	Pyruvate dehydrogenase	MS/MS		O00330	ATP production, glycolysis
88*†	E-3 binding protein	MS/MS			
89*†	Myosin light chain 2	MALDI	18	P10916	Muscle contraction
90*†			16		
91*†	Myosin light chain 1	MS/MS		P08590	
92*†	Myosin light chain 1	MALDI	59	P08590	
93	Alpha-crystallin B chain	MALDI	44	P23927	Organizational chaperone
94	Enoyl-CoA hydratase	MALDI	23	P14604	ATP production, beta oxidation
95	D-beta-hydroxybutyrate dehydrogenase	MALDI	28	P29147	ATP production, oxidative phosphorylation
96	Troponin T, cardiac	MALDI	18	P50751	Muscle contraction
97	Acyl-CoA dehydrogenase, short chain specific	MALDI	18	P15651	ATP production, beta-oxidation
98	Acyl-CoA dehydrogenase, long chain specific	MALDI	22	P15650	ATP production, beta-oxidation
99*†	ATP synthase beta chain	MALDI	49	P10719	ATP production, oxidative phosphorylation

100	Creatine kinase M chain	MALDI	47	P00563	ATP regeneration
101	Succinyl-CoA:3 ketoacid coenzyme A transferase	MALDI	20	P55809	ketone body catabolism
102*	Dihydrolipoamide	MALDI	45	P49819	ATP production, the TCA acid cycle
103*	dehydrogenase		18		
104*†	Fumarate hydratase	MALDI	31	P07954	
105*†			28		
106	Beta enolase	MALDI	18	P25704	ATP production, glycolysis
107*†	Fumarate hydratase	MALDI	15	P07954	ATP production, the TCA acid cycle
108*	Succinate dehydrogenase	MALDI	18	P31040	
109*	[ubiquinone] flavoprotein		23		
110*	subunit		14		
111*			38		
112	Heat shock protein 60	MALDI	35	P19226	Stress response

This revealed that while the adenosine- and diazoxide-induced protein changes identified here occurred to TCA cycle, OxPhos, chaperone proteins, Ca²⁺ handling proteins, and to proteins selected from aldehyde dehydrogenase, NG-dimethylarginine dimethylaminohydrolase (DDAH) and the RNA binding protein regulatory subunit DJ-1, there were also additional proteins not detectably modified by preconditioning in these experiments (see Figure 6) and thus not identified as preconditioning proteins as defined herein.

The identification of the preconditioning proteins described herein has provided a means for identifying and using compositions and methods for modulating these preconditioning proteins and priming cells for preconditioning and/or inducing and/or modulating preconditioning of a cell, tissue or organ.

Accordingly, an aspect of the present invention relates to compositions and methods or events for modulating a preconditioning protein such as the abundance of a TCA cycle enzyme and/or an OxPhos component and/or a chaperone protein and/or a Ca²⁺ handling protein and/or a protein selected from aldehyde dehydrogenase, NG-dimethylarginine dimethylaminohydrolase (DDAH) and the RNA binding protein regulatory subunit DJ-1 in cells by administering to the cells compositions or subjecting the cells to a method or event which is known to induce preconditioning of organs such as the heart, skeletal

muscle, smooth muscle, brain, kidney and/or liver. For example, as shown herein, preconditioning proteins are modulated by treating cells with a pharmacological preconditioning agent. It is expected that these
5 preconditioning proteins can be modulated similarly by exposing the cells to an event such as brief ischemic or hypoxic episode. Modulations in preconditioning proteins expected with the compositions, methods and/or events of the present invention include, but are not limited to one
10 or more of the following changes:

an increase in level of IDH; succinyl CoA ligase; the 23 kDa subunit, 24 kDa subunit, and/or 30 kDa subunit (mitochondrial precursors) of Complex I; the δ chain (mitochondrial precursor) of the F_1 portion, and/or the d
15 chain (mitochondrial precursor) of the F_0 portion of Complex V; prohibitin; ADP ribosyl hydrolase; HSP27; and/or the RNA binding protein regulatory subunit (DJ-1);

a decrease in level of dihydrolipoamide succinyltransferase; core protein I of Complex III; metaxin
20 2; and/or sarcalumenin;

a change (increase or decrease) in the level of DDAH; and/or

an increase in the post-translational modification of β chain (mitochondrial precursor) of the F_1 portion of
25 Complex V; protein X; and/or aconitate hydratase (aconitase).

Another aspect of the present invention relates to methods for the identification of new compositions and methods or events useful in modulating a preconditioning
30 protein and/or in priming a cell for preconditioning and/or inducing or modulating preconditioning of a cell, tissue organ. Such new compositions, methods or events can be identified routinely in accordance with the teachings herein based upon their ability to modulate one or more of
35 the preconditioning proteins identified herein. Modulation of one or more of the preconditioning proteins can be assessed in a cell, tissue or organ by detection of one or more of the proteins in the presence and absence of the

composition, method or event. A change in the abundance or partitioning of the protein itself and/or post-translational product of the protein in the cell, tissue or organ in the presence of the composition, upon exposure of the cell, tissue or organ to the method or event, as compared to a cell, tissue or organ in the absence of the composition or a cell, tissue or organ not exposed to the method or event is indicative of the composition, method or event modulating the preconditioning protein and/or priming the cell for preconditioning and/or inducing or modulating preconditioning of a cell, tissue or organ. Preferred compositions, methods or events of the present invention will produce one or more of the following changes:

an increase in level of IDH; succinyl CoA ligase; the 23 kDa subunit, 24 kDa subunit, and/or 30 kDa subunit (mitochondrial precursors) of Complex I; the δ chain (mitochondrial precursor) of the F_1 portion, and/or the d chain (mitochondrial precursor) of the F_0 portion of Complex V; prohibitin; ADP ribosyl hydrolase; HSP27; and/or the RNA binding protein regulatory subunit (DJ-1);

a decrease in level of dihydrolipoamide succinyltransferase; core protein I of Complex III; metaxin 2; and/or sarcalumenin;

a change (increase or decrease) in the level of DDAH; and/or

an increase in the post-translational modification of β chain (mitochondrial precursor) of the F_1 portion of Complex V; protein X; and/or aconitate hydratase (aconitase).

More preferred are compositions, methods or events that mimic the modulation of the preconditioning proteins by adenosine or diazoxide. Most preferred are compositions comprising small organic molecules. Such small organic molecules can be designed to have similar structure and therefore similar activity to adenosine or diazoxide. Alternatively, screening assays for small organic molecules with similar function to adenosine or diazoxide in

modulating preconditioning proteins in cells can be used to identify compositions of the present invention.

Compositions, methods and/or events identified as modulating a preconditioning protein are expected to be
5 useful in priming a cell for preconditioning and/or as preconditioning agents. Accordingly, another aspect of the present invention relates to regulation of a preconditioning protein as a means for priming cells for preconditioning and/or preconditioning a cell, tissue or
10 organ and preventing cell injury and/or cell death upon an ischemic and/or hypoxic episode. As each of the preconditioning proteins are involved in important cellular processes, compositions, methods and/or events can also be used to monitor and/or modulate one or more of the cellular
15 processes, i.e. TCA cycling, oxidative phosphorylation, Ca^{2+} handling, chaperones, and/or modulating a protein selected from aldehyde dehydrogenase, NG-dimethylarginine dimethylaminohydrolase (DDAH) and the RNA binding protein regulatory subunit DJ-1. Understanding the effects of
20 modulation of the preconditioning proteins and/or the cellular processes in which they are involved will lead to better treatment of patients suffering from cell injury or cell death such as that caused by ischemia-reperfusion or hypoxic-reperfusion injury. For example, following cardiac
25 arrest during surgery there are little or no free nucleotides left in myocytes and acidity (hydrogen ion content) of the cells is increased. In some cases, adenosine is added to stimulate ATP synthesis. This may only aid in the short term if adenosine also causes a
30 reduction in the quantity of the functioning F_1F_0 ATPase in the mitochondria with time (time being required for the modified β -chain to be incorporated into the mature complex). Long term treatment thus may require blocking or eliminating adenosine action subsequent to obtaining its
35 beneficial short term effects.

Alternatively, if modulation of TCA cycle enzymes and/or OxPhos proteins and/or chaperone proteins and/or Ca^{2+} handling proteins and/or proteins selected from aldehyde

dehydrogenase, NG-dimethylarginine dimethylaminohydrolase (DDAH) and the RNA binding protein regulatory subunit DJ-1 are demonstrated to be beneficial in that they enhance or reduce the activity of oxidative metabolism during a subsequent ischemia such that during reperfusion following ischemia the extent of cell death via necrosis and/or apoptosis is reduced, then further promotion of the modifications via administration of additional adenosine or diazoxide (or equivalent agents) may be desired.

Further, as shown herein, different pharmacological preconditioning agents caused different modifications of these preconditioning proteins. For example, pharmacological preconditioning with diazoxide resulted in an increase in IDH; the 23, 24, and 30 kDa subunits (mitochondrial precursors) of Complex I; the δ chain (mitochondrial precursor) of the F_1 portion, and the d chain (mitochondrial precursor) of the F_0 portion of Complex V; ADP ribosyl hydrolase; DDAH; HSP27; RNA binding protein regulatory subunit (DJ-1); and post-translational modification of protein X; and a decrease in core protein I of Complex III; dihydrolipoamide succinyltransferase; metaxin 2; and sarcalumenin. Pharmacological preconditioning with adenosine resulted in an increase in IDH; succinyl CoA ligase; the 24 and 30 kDa subunits (mitochondrial precursors) of Complex I; the δ chain (mitochondrial precursor) of the F_1 portion of Complex V; and the post-translational modification of the β chain (mitochondrial precursor) of the F_1 portion of Complex V; prohibitin; ADP ribosyl hydrolase; and RNA binding protein regulatory subunit (DJ-1); and a decrease in core protein I of Complex III; metaxin 2; and DDAH. Accordingly, preconditioning agents may be sub-categorized based upon their ability to modulate different preconditioning proteins identified herein. Such sub-categorization will be useful in selecting varying treatment regimes, particularly for patients on long-term therapy wherein desensitization to a single preconditioning agent is oftentimes observed. Accordingly, individuals at greater

risk of an ischemic event can be maintained in a long-term preconditioned state without desensitizing them to preconditioning agents, by selectively administering different subcategories of preconditioning agents that, while having the same end effect of protecting cells, tissues or organs from death, do not necessarily act on the exact same proteins to confer this protection.

Another aspect of the present invention relates to methods for diagnosing and/or monitoring in a subject preconditioning and/or ischemic, hypoxic, ischemic/reperfusion or hypoxic/reperfusion conditions and/or the ability of a cell, tissue or organ to survive injury by monitoring modulation of one or more of the preconditioning proteins. One or more of the preconditioning proteins may be detected in a sample of injured cells, tissue or organ as well as in a biological fluid such as, for example, blood, serum, plasma, urine, bile, saliva, semen, mucus or cerebrospinal fluid, obtained from the subject.

In one embodiment of this aspect of the present invention, levels of a preconditioning protein can be monitored in a subject to assess whether a cell, tissue or organ has been subjected to sufficient preconditioning or requires additional preconditioning for protection from cell, tissue or organ injury or death.

Diagnosis of an ischemic or hypoxic condition can also be performed by comparing levels of a preconditioning protein measured in a subject with levels of this protein in a control. A difference in levels of a preconditioning protein in the subject as compared to the control is indicative of an ischemic or hypoxic condition in the subject. Differences in preconditioning proteins diagnostic of an ischemic or hypoxic condition include:

an increase in level of IDH; succinyl CoA ligase; the 23 kDa subunit, 24 kDa subunit, and/or 30 kDa subunit (mitochondrial precursors) of Complex I; the δ chain (mitochondrial precursor) of the F_1 portion, and/or the d chain (mitochondrial precursor) of the F_0 portion of Complex

V; prohibitin; ADP ribosyl hydrolase; HSP27; and/or the RNA binding protein regulatory subunit (DJ-1);

a decrease in level of dihydrolipoamide succinyltransferase; core protein I of Complex III; metaxin
5 2; and/or sarcalumenin;

a change (increase or decrease) in the level of DDAH; and/or

an increase in the post-translational modification of β chain (mitochondrial precursor) of the F_1 portion of
10 Complex V; protein X; and/or aconitate hydratase (aconitase).

As used herein, by "control" it is meant, a sample obtained from an individual known not have an ischemic or hypoxic condition, a sample obtained previously from the
15 subject prior to the onset or suspicion of the ischemic or hypoxic condition, or a standard from data obtained from a data bank corresponding to currently accepted normal levels of the preconditioning protein. One or more of: an increase in level of IDH; succinyl CoA ligase; the 23 kDa
20 subunit, 24 kDa subunit, and/or 30 kDa subunit (mitochondrial precursors) of Complex I; the δ chain (mitochondrial precursor) of the F_1 portion, and/or the d chain (mitochondrial precursor) of the F_0 portion of Complex V; prohibitin; ADP ribosyl hydrolase; HSP27; and/or the RNA
25 binding protein regulatory subunit (DJ-1); a decrease in level of dihydrolipoamide succinyltransferase; core protein I of Complex III; metaxin 2; and/or sarcalumenin; a change (increase or decrease) in the level of DDAH; and/or an increase in the post-translational modification of β chain
30 (mitochondrial precursor) of the F_1 portion of Complex V; protein X; and/or aconitate hydratase (aconitase) are indicative of the subject having an ischemic or hypoxic condition. The comparison performed may be a straight-forward comparison, such as a ratio, or it may involve
35 weighting of one or more of the measures relative to, for example, their importance to the particular situation under consideration. The comparison may also involve subjecting

the measurement data to any appropriate statistical analysis.

The following nonlimiting examples are provided to further illustrate the present invention.

EXAMPLES**Example 1: Isolation and Preconditioning of Rabbit Ventricular Myocytes**

Ventricular myocytes from New Zealand White rabbits
5 (weighing 1 to 2 kg) were isolated by collagenase
dissociation, as described previously by Liu et al. (Circ.
Res. 1996 78:443-454). Hearts were excised, then perfused
with collagenase (1.0 mg/mL, Worthington type II) for 14
minutes at a maintained perfusion pressure of 75 mm Hg on a
10 Langendorff apparatus, yielding >50% Ca²⁺-tolerant
ventricular myocytes. Cell isolation was followed directly
by pharmacological preconditioning, which was carried out
by treatment with 100 µmol/L adenosine (Sigma) or with 100
µmol/L diazoxide (Sigma) for 60 minutes in a 37° C water
15 bath, as described previously by Liu et al. *supra*.
Untreated cells were prepared concurrently as drug-free
controls. Equivalent 25 µL aliquots of cells (containing
~30 mg/mL of protein as determined by Lowry assay (Lowry,
O.H. J. Biol. Chem. 1951 193:265-275) were frozen and
20 stored at -80° C until analysis.

Example 2: In Sequence Protein Extraction and Subcellular Fractionation

All steps in this Protein extraction protocol,
25 referred to herein as "In Sequence" and depicted in Figure
3, produce physiological pH (cytosolic) and acidic pH
enriched extracts were carried out at 4° C, and all
centrifugations were conducted at 16000xg for 2 minutes at
4° C. Myocyte proteins were first extracted by two rounds
30 of homogenization in 100 µL of HEPES extraction buffer,
consisting of (in mmol/L) HEPES 25 (pH 7.4), NaF 50, Na₃VO₄
0.25, phenylmethylsulfonyl fluoride 0.25, EDTA 0.5, and (in
µmol/L) leupeptin 1.25, pepstatin A 1.25. Following
homogenization and centrifugation, the supernatants were
35 pooled and saved as the cytosolic extract. The remaining
pellet was subjected to further extraction by two rounds of
homogenization in 50 µL of acid extraction buffer,
consisting of 1% v/v trifluoroacetic acid (TFA) and 1

mmol/L Tris (2-carboxyethylphosphine) hydrochloride (pH ~2.0). Supernatants were again pooled, and saved as the acid extract. The two extracts and remaining pellet were frozen and stored at -80° C.

5

Example 3: Two-Dimensional Gel Electrophoresis (2-DE)

Isoelectric focusing (IEF) of cytosolic and TFA extracts were carried out using a Protean® IEF cell (Bio-Rad) according to the manufacturer's protocol. Protein loads ranging from 5 up to 250 µg per gel were added to the following rehydration buffers: for cytosolic extracts, 8 M urea, 2.5 M thiourea, 4% CHAPS, 2 mM EDTA, and 25 mM DTT, and in the case of pH 6-9 strips, CHAPS and DTT were substituted by 4% ASB-14 and 5.4% (w/v) 2-hydroxyethyl disulfide, respectively; for acidic extracts, 8 M urea, 4% CHAPS, and 25 mM DTT. Immobilized pH gradient (IPG) Ready Strips™ (170 mm pH 4-7, 6-9, or 3-10 linear gradient, Bio-Rad) were actively rehydrated at 50 volts (V) for 10 hours to enhance protein uptake, then subjected to the following conditions using a rapid voltage ramping method: 100 V for 25 Volt-hours (Vh), 500 V for 125 Vh, 1000 V for 250 Vh, and 8000 V for 65 kVh. A Peltier temperature control platform maintained gels at 20° C throughout IEF. Focused gels were stored at -20° C prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

For SDS-PAGE, IPG strips were incubated for 10 minutes in equilibration buffer (50 mmol/L Tris-HCl, pH 8.8, 6 mol/L urea, 30% v/v glycerol, 2% w/v SDS) supplemented with 10 mg/mL DTT, followed by a 10 minute incubation in equilibration buffer supplemented with 25 mg/mL iodoacetamide, then rinsed once with SDS-PAGE buffer (25 mmol/L Tris, 192 mmol/L glycine, pH 8.3, 0.1% w/v SDS). IEF strips were then embedded in a 5% acrylamide stacking gel and the proteins were resolved by 10 or 12.5% SDS-PAGE using a Protean® II XL system (Bio-Rad). Electrophoresis was carried out until the dye front reached the bottom of the resolving gel (50 V for 30 minutes until proteins were transferred from the IPG strips to the stacking gel,

35

followed either by 150 V for 7.5 hours or by 250 V for 4 hours).

Example 4: Protein Transfer and Western Blotting

5 Following 2-DE, gels were equilibrated in SDS-PAGE buffer supplemented to 20% v/v methanol for 10 minutes, then transferred in the same buffer to nitrocellulose at 200 mA constant current for 2 hours. Nitrocellulose membranes were then rinsed with phosphate-buffered
10 saline/Tween-20 (PBS/T), consisting of (in mmol/L) NaCl 137, KCl 2.7, Na₂HPO₄ 10.1, KH₂PO₄ 1.8, pH 7.4 supplemented to 0.1% v/v Tween-20, then blocked overnight at 4° C with 1% v/v blocking reagent (Roche Diagnostics) in PBS/T. Western blotting for ATP synthase β -chain was performed at 1 μ g/mL
15 with the anti-ATP synthase β -chain antibody Clone No. 7E3-F2 (Molecular Probes Cat. No. A-21299, Eugene, Oregon), and detected by chemiluminescence with an alkaline phosphatase-conjugated secondary antibody.

20 **Example 5: Silver Staining of Two-Dimensional Gels**

Two-dimensional gels were silver stained according to the protocol of Shevchenko et al. (Anal. Chem. 1996 68:850-858) for compatibility with subsequent analysis of proteins by mass spectrometry. Gels were fixed overnight in 50% v/v
25 methanol, 5% v/v acetic acid, followed by 50% v/v methanol for 10 minutes, then 10 minutes in deionized distilled (dd) H₂O. Gels were sensitized for 1 minute in 0.02% w/v sodium thiosulfate, followed by two 1-minute ddH₂O washes, then incubated in chilled (4° C) 0.1% w/v silver nitrate for 20
30 minutes, followed again by two 1-minute ddH₂O washes. Proteins were then visualized by several washes with developing solution (2% w/v sodium carbonate, 0.04% v/v formalin) until maximum staining was obtained without a concomitant increase in background staining, after which

development was stopped with 5% v/v acetic acid.

Example 6: Image Analysis and Quantification

Silver-stained 2-D gels were digitized at 150 dpi
5 (pixels per inch) resolution using a PowerLookII® scanner
(UMAX® Data Systems, Inc.) on a Sun® Ultra5™ computer (Sun
Microsystems, Inc.). Protein spots were then located,
quantified, and matched to spots on other gels using
Investigator™ HT Proteome Analyzer 1.0.1 software (Genomic
10 Solutions, Inc.). A number of manually defined spots were
selected as anchors (preferably 15 or more) for
triangulation of remaining spots. Composite images were
then prepared by matching spots from four gel images for
each treatment group (adenosine, diazoxide, and control).
15 Protein spot normalization (for n=4) was carried out by
using total spot intensity ratio to normalize gel staining
across treatments and animals, or by using a match ratio
method for determination of the extent of modification for
protein spots from post-translationally modified proteins.

20

Example 7: Mass Spectrometry

Protein Preparation

Protein spots extracted from 2-D gels were destained
25 according to Gharahdaghi et al. (Electrophoresis 1999
20:601-605), then dried under vacuum before enzymatic
digestion with sequence-grade modified trypsin (Promega) or
Asp-N (Sigma). Peptides were extracted with 50%
acetonitrile (ACN)/5% TFA, dried under vacuum, and
30 reconstituted with 3 µL of 50% ACN/0.1% TFA. Reconstituted
extract (0.5 µL) was mixed with 0.5 µL of matrix (10 mg/mL
α-cyano-4-hydroxy-trans-cinnamic acid in 50% ACN, 0.1%
TFA); spotted on a stainless steel 100-well mass
spectrometry plate, and air-dried.

35

MALDI-TOF MS of Cytosolic and Mitochondrial Proteins:

Samples were analyzed using a Voyager® DE-Pro matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (PerSeptive Biosystems) reflector
5 equipped with a 337 nm nitrogen laser operated in the delayed extraction/reflector mode with an accelerating voltage of 20 kV, grid voltage setting of 72%, and a 50 ns delay. Five spectra (50-100 laser shots/spectrum) were obtained for each sample. External calibration was
10 performed using a Sequazyme Peptide Mass Standard kit (PerSeptive Biosystems) containing the following standards: des-Arg-bradykinin, angiotensin-1, and Glu-fibrinopeptide B.

15 MALDI-QTOF MS/MS:

MALDI MS/MS spectra were collected on an Applied Biosystems/MDS-Sciex QSTAR pulsar QTOF instrument (Concord, Ontario, Canada) equipped with an orthogonal MALDI source employing a 337 nm nitrogen laser. The instrument was
20 operated in positive mode and collision-induced dissociation (CID) of peptides was achieved with argon as the collision gas. Spectra were acquired and processed using Sciex support software.

25 Example 8: Bioinformatic Data Analysis

Peptide mass fingerprinting was conducted with the database search tool MS-Fit in the program Protein Prospector, to search the Swiss-Prot protein database. A number of restrictions were applied to the search: species
30 = mammals, pI range variable (depending on spot of interest), mass range variable (usually with 50-100 ppm mass tolerance), with a minimum of 4 peptides to match, and a maximum of one missed tryptic or Asp-N cleavage, with possible modifications including Cys-carbamidomethylation,
35 Met-oxidation, protein N-terminal acetylation, and acrylamide modified Cys.